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(54) Title: METHOD FOR PREPARING RADIONUCLIDE-LABELED CHELATING AGENT-LIGAND COMPLEXES

(57) Abstract

Radionuclide-labeled chelating agent-ligand complexes that are useful in medical diagnosis or therapy are prepared by reacting a radionuclide, such as ^{90}Y or ^{111}In, with a polyfunctional chelating agent to form a radionuclide chelate that is electrically neutral; purifying the chelate by union exchange chromatography; and reacting the purified chelate with a targeting molecule, such as a monoclonal antibody, to form the complex.

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METHOD FOR PREPARING RADIONUCLIDE-LABELED CHELATING AGENT-LIGAND COMPLEXES

Reference to Government Support

This invention was made in part with Government support under contract number CA16861 and CA47829 awarded by the Department of Health and Human Services and contract number DE FG03-84ER60233 awarded by the Department of Energy. The Government has certain rights in this invention.

Description

Technical Field

This invention relates to a method for preparing radionuclide-containing compounds that are useful for medical diagnosis and therapy.

Background Art

Macrocyclic bifunctional chelating agents have been developed to tag monoclonal antibodies (mAbs) with radiometals for *in vivo* diagnosis and therapy [Moi *et al.*, 1985, Anal. Biochem., 148:249-253; Moi *et al.*, 1988, J. Am. Chem. Soc., 110:6266-6267; Cox *et al.*, 1990, J. Chem. Soc. Perkins Trans. 1, 2567-2576; Parker, 1990, Chem. Soc. Rev., 19:271-291; Meares *et al.*, 1990, British J. Cancer, Suppl., 10:21-26; Gansow, 1991, Nucl. Med. Biol., 18:369-381; Li *et al.*, 1993, Bioconjugate Chem., 4:275-283]. In particular, mAbs labeled with DOTA (1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid) derivatives incorporating yttrium-90 (^{90}Y) and indium-111 (^{111}In) have shown excellent kinetic stability under physiological conditions [Moi *et al.*, 1988; Meares *et al.*, 1990; Li *et al.*, 1993; Deshpande *et al.*, 1990, J. Nucl. Med., 31:473-479]. However, the slow formation of yttrium-DOTA complexes [Kapryzyk *et al.*, 1982, Inorg. Chem., 21:3349-3352; Kodama *et al.*, 1991, Inorg. Chem., 30:1270-1273; Wang *et al.*, 1992, Inorg. Chem., 31:1095-

1099] presents a technical problem that can lead to low radiolabeling yields unless conditions are carefully controlled.

These chelating agent-mAb-radionuclide conjugates have been synthesized using two methods. In one, the chelating agent is first conjugated to the antibody and then the resulting conjugate is labeled with radionuclide. In the other, called "prelabeling", the chelating agent is first labeled with the radionuclide, the labeled chelating agent is purified, and the purified labeled chelating agent is conjugated to the antibody. Prelabeling has several potential advantages over the other method. In the labeling step, metal chelate formation is easier to control because there is no competition from metal binding sites on the mAb and there is no danger of denaturing the antibody during labeling. The removal of unreacted chelating agent in the purification step avoids the production of multiply labeled immunoconjugates with unfavorable biological properties. Finally, prelabeling minimizes chemical manipulation of the antibody and reduces loss of antibody activity.

Prelabeling has been used to label mAbs with ^{99}Tc [Fritzberg *et al.*, 1987, Proc. Natl. Acad. Sci. U.S.A., 85:4025-4029; Franz *et al.*, 1987, Nucl. Med. Biol., 14:569-572; Linder *et al.*, 1991, Bioconjugate Chem., 2:160-170], ^{67}Cu [Moi *et al.*, 1985, *supra*] and ^{177}Lu [Schlom *et al.*, 1991, Cancer Res., 51:2889-2896]. It has not heretofore been used to label mAbs with ^{90}Y or ^{111}In.

In these prior instances of prelabeling, the metal chelate has either not been purified prior to conjugation [Moi *et al.*, 1985, *supra*] or has been purified by HPLC [Fritzberg *et al.*, 1987; Schlom *et al.*, 1991, *supra*]. It is noted that Moi *et al.* use anion exchange chromatography to characterize their chelate (as a divalent anion) but not to purify it. The use of HPLC is

not desirable because it employs mixed aqueous/organic solvents for elutions.

Disclosure of the Invention

The present invention applies the prelabeling process to ^{90}Y and ^{111}indium labeling and provides a prelabeling process that employs anion exchange chromatography to purify the radionuclide chelate.

Accordingly, one aspect of the invention is a method for preparing a yttrium- or indium-labeled chelating agent-ligand complex comprising:

(a) reacting a chelating agent that has a trivalent chelating group and at least one pendant linker group that is capable of covalently binding to a ligand, with yttrium-90 or indium-111 to form an electrically neutral yttrium-90 or indium-111 chelate;

(b) purifying the chelate from the reaction mixture of step (a); and

(c) reacting the purified chelate of step (b) with the ligand to form said complex.

Another aspect of the invention is a method for preparing a radionuclide-labeled chelating agent-ligand complex comprising:

(a) reacting a chelating agent that has a chelating group and at least one pendant linker group that is capable of covalently binding a ligand, with a radionuclide to form a radionuclide chelate;

(b) purifying the radionuclide chelate from the reaction mixture of step (a) by anion exchange chromatography; and

(c) reacting the purified radionuclide chelate of step (b) with the ligand to form said complex.

Brief Description of the Drawings

Figure 1 is a flow chart that illustrates the synthetic route used for the preparation of radionuclide-labeled chelating ligand complexes.

Figure 2 is a graph depicting the biodistribution of ^{90}Y-DOTA-Gly$_3$-L-Phe-amide-thiourea-chimeric mAb L6 (compound (3) of Figure 1) in HBT tumor-bearing nude mice at day 1, day 3, and day 5. For each time point, data were acquired from 7 animals. The values are given as average percent of injected dose per gram of tissue. Error bars represent 1 standard deviation.

Detailed Description of the Invention

A. The novel prelabeling method

Prelabeling involves three steps: (1) formation of a radiolabeled chelate (in the absence of ligand(s)), (2) purification of the radiolabeled chelate, and (3) conjugation of the purified radiolabeled chelate with ligand(s) to form a radiolabeled chelating agent-ligand complex.

The medically useful radiometals which are important for practical applications have short half-lives [Wessels *et al.*, 1984, Med. Phys., 11:638-645] and high efficiencies of both labeling and conjugation. The prelabeling approach permits use of a large excess of chelating agent to achieve a high chelation yield quickly in step (1), but requires a rapid purification method to remove unlabeled reagent in step (2). The present invention provides an easy and efficient method for prelabeling a chelating agent (for example, a peptide-linked DOTA derivative) with a radiometal (for example, ^{90}Y or ^{111}In) and subsequently conjugating it to a targeting molecule (for example, a mAb).

In the conventional labeling method, the number of radionuclide chelating moieties attached to each targeting molecule is usually >1 in order to provide enough chelating groups for a good radiolabeling yield.

However, the chelating groups that actually chelate radionuclides comprise less than 5% of the total attached chelating groups on the targeting molecule. The excess chelating groups may affect the biological properties of the targeting molecule, e.g. by inducing an immune response [Kosmas *et al.*, 1992, *supra*], and impure metal solutions may require large amounts of the targeting molecule.

With prelabeling, a far smaller number of chelates are attached to the targeting moiety, but practically all are radiolabeled; thus the number of modified targeting molecules is significantly reduced, and the number of multiply-modified targeting molecules is essentially zero. For example, when the targeting molecule is a mAb, the radiolabeled mAbs are fully immunoreactive and are expected to have more favorable biological properties, including reduced immunogenicity.

B. Formation of the radiolabeled chelate

The first step of prelabeling involves the formation of a radiolabeled-chelating agent (also referred to as a radiolabeled chelate) from a chelating agent and an appropriate radionuclide.

Synthetic methods for the preparation of chelating agents useful in the practice of the invention are known in art [see, for example Li *et al.*, 1993, *supra*].

Methods for the preparation of radiolabeled chelates by reaction of a radionuclide with a chelating agent are known in the art [see, for example, Moi *et al.*, 1985, *supra*]. Typically, the chelating agent is dissolved in a buffered aqueous medium and the purified radionuclide added. The pH may be selected to optimize conditions for chelate formation. For example, when chelation is achieved by acetate groups binding to the metal ion (as is the case for various acetic acid compounds), the pH may be adjusted (using, for example, aqueous tetramethylammonium acetate, to obtain of pH of about 3

to about 6, more preferably about 5) to provide a preponderance of ionized carboxylate ($-COO^-$) groups, and thereby yield a chelating species which is anionic. Furthermore, the reaction mixture temperature may be adjusted, for example to 37°C for 30 min, to accelerate the reaction (chelation). After a period of time or upon completion of reaction, an excess of an appropriate quenching agent, such as DTPA may be added. The quenching agent acts to form anionic quenching chelates with any radionuclide not yet chelated by the chelating agent. The resulting reaction mixture may then be purified by the second step of prelabeling.

The term "radionuclide", as used herein, relates to medically useful radionuclides, including, for example, positively charged ions of radiometals such as Y, In, Cu, Lu, Tc, Re, Co, Fe and the like, such as ^{90}Y, ^{111}In, ^{67}Cu, ^{177}Lu, ^{99}Tc and the like, preferably trivalent cations, such as ^{90}Y and ^{111}In.

The term "chelating agent", as used herein, relates to polyfunctional compounds have a chelating group and at least one pendant linker group, wherein the chelating group is capable of chelating with a radionuclide, and the pendant linker group(s) is capable of covalently binding to one or more targeting molecules which may be the same or different. Chelating agents may be represented by the formula $A(L)_n$ wherein A represents the chelating moiety, L represents a pendant linking group, and n is an integer from 1 to 3, preferably 1. The pendant linker group includes one or more functional group(s) which are capable of covalently binding to targeting molecule(s).

Chelating groups capable of chelating radionuclides include macrocycles, linear, or branched moieties. Examples of macrocyclic chelating moieties include polyaza- and polyoxamacrocycles. Examples of polyazamacrocyclic moieties include those derived from compounds such at 1,4,7,10-tetraazacyclododecane-

N,N',N'',N'''-tetraacetic acid (herein abbreviated as DOTA); 1,4,7,10-tetraazacyclotridecane-N,N',N'',N'''-tetraacetic acid (herein abbreviated as TRITA); 1,4,8,11-tetraazacyclotetradecane-N,N',N'',N'''-tetraacetic acid (herein abbreviated as TETA); and 1,5,9,13-tetraazacyclohexadecane-N,N',N'',N'''-tetraacetic acid (abbreviated herein abbreviated as HETA). Examples of linear or branched chelating moieties include those derived from compounds such as ethylenediaminetetraacetic acid (herein abbreviated as EDTA) and diethylenetriaminepentaacetic acid (herein abbreviated as DTPA).

Chelating moieties having carboxylic acid groups, such as DOTA, TRITA, HETA, HEXA, EDTA, and DTPA, may be derivatized to convert one or more carboxylic acid groups to amide groups.

The term "pendant linker group", as used herein, relates to moieties which are attached to the chelating group, and which have at least one functional group which is capable of covalently binding to targeting molecules. Where pendant linkers or chelating agents have a plurality of such functional groups, they may be the same or different. When the chelating moiety is macrocyclic, the pendant moiety may be attached to any annular atom. For example, when the chelating moiety is a polyazamacrocycle, the pendant group may be attached to an annular carbon atom or an annular nitrogen atom. When the pendant group is attached to an annular nitrogen atom, the compound may be referred to as an N-substituted polyazamacrocycle.

The term "functional groups capable of covalently binding to targeting molecules", as used herein, includes those functional groups which can be activated by known methods, so as to be capable of covalently binding to targeting molecule(s); for example, the formation of active esters (-C(=O)OR, wherein R is, for example, succinimidyl) from carboxylic acids, the formation of

acid halides (-C(=O)X, wherein X is typically Cl or Br) from carboxylic acids.

The functional group(s) present on the pendant linker group which are capable of covalently binding to targeting molecules may be chosen according to the targeting molecule(s) to which the chelating agent will ultimately be bound. Reactive pairs of functional groups permit conjugation of the chelating agent with the targeting molecule, via the linker group, wherein one member of the pair is present on the chelating agent and the other member of the pair is present on the targeting molecule. For example, when the targeting molecule is a protein possessing a free amino ($-NH_2$) group, a functional group such as isothiocyanate (-NCS) present on the chelating agent permits reaction to form a joining linkage (in this case, a thiourea linkage), thereby forming a chelating agent-targeting molecule complex. Other examples of appropriate reactive pairs of functional groups include, for example, $-NH_2$ with -C(=O)OR (active ester) or with -C(=O)OC(=O)R (anhydride) or with -C(=O)X (acid halide) to yield an amide linkage; $-NH_2$ with -NCO (isocyanate) to yield a urea linkage. Other reactive pairs involving $-NH_2$ include $-NH_2$ and $-S(=O)_2X$ (sulfonyl halide); $-NH_2$ and -C(=NR)OR (imidate ester); and $-NH_2$ and -OC(=O)X (haloformate). Examples of reactive pairs of functional groups include -SH and $-C(=O)CH_2X$ (haloacetyl) to yield a $-SCH_2C(=O)-$ linkage; -SH and -alkyl-X (alkyl halide) or -SH and -S(=O)O-alkyl (alkyl sulfonate) to yield a thioether; and -SH and -SH (sulfhydryl) to yield a -SS- (disulfide) linkage.

Li *et al.* [Li *et al.*, 1993, *supra*] have shown that the introduction of a cleavable linker between the chelate and the mAb results in a reduction of accumulated radioactivity in the liver [Li *et al.*, 1993, *supra*]. Preferably, radionuclide-labeled chelating agent-ligand complexes will be cleavable *in vivo*. This may be achieved by introducing a cleavable linkage within the

pendant group, wherein the cleavable linkage is cleaved *in vivo*, for example, by enzymatic action within the liver. Examples of such cleavable linkages include peptide, disulfide, and ester linkages.

Examples of pendant linker groups include peptide-based linkers, such as polypeptide groups which have been derivatized to possess at least one functional group capable of covalently binding to targeting molecule(s). The number of peptide linkages present in the pendant linker group may be varied to optimize radionuclide chelation, conjugation with targeting molecule(s), *in vivo* cleavability, or other factors. Examples of suitable pendant linker groups include $-CH_2-C(=O)(AA)_m(AA-FG)$, herein denoted as substituted acetyls, wherein the $-CH_2-C(=O)-$ fragment may be derived from an acetate moiety, AA represents an amino acid diradical, more preferably the glycine diradical $-NH-CH_2-C(=O)-$, and m is an integer, preferably between 1 and 10, more preferably between 3 and 7, most preferably 3. AA-FG represents an amino acid N-radical (that is, the free bond is situated on the amino group of the amino acid) which has been derivatized to possess a functional group (FG) capable of covalently binding to targeting molecule(s). Preferably, the carboxylic acid group of the amino acid of AA-FG has been derivatized, for example, to form an amide. Examples of AA-FG radicals include *p*-isothioscyanato-phenylalanine-N-yl amide ($-NHCH[C(=O)NH_2][CH_2-(p\text{-}NCS-C_6H_4)]$ denoted herein as *p*-NCS-Phe-amide, or *p*-NCS-L-Phe-amide). Examples of pendant groups include $-CH_2-C(=O)(Gly)_m(p\text{-}NCS\text{-}Phe\text{-}amide)$, denoted herein as Gly_m(*p*-NCS-Phe-amide)acetyl. Further examples of pendant linker groups include disulfides, such as alkyl disulfides including $-CH_2-C(=O)-(CH_2)_pSS(CH_2)_qNS$ and the like, and esters, such as $-CH_2-C(=O)-(CH_2)_pC(=O)O(CH_2)_qNSC$ and the like, wherein p and q are integers from about 1 to about 8.

Examples of chelating agents include:

1,4,7,10-tetraazacyclododecane-N-(Gly_3(*p*-NCS-Phe-amide)acetyl)-N',N'',N'''-triacetic acid;
1,4,7,10-tetraazacyclotridecane-N-(Gly_3(*p*-NCS-Phe-amide)acetyl)-N',N'',N'''-triacetic acid;
1,4,8,11-tetraazacyclotetradecane-N-(Gly_3(*p*-NCS-Phe-amide)acetyl)-N',N'',N'''-triacetic acid;
1,5,9,13-tetraazacyclohexadecane-N-(Gly_3(*p*-NCS-Phe-amide)acetyl)-N',N'',N'''-triacetic acid;
ethylenediamine-N-(Gly_3(*p*-NCS-Phe-amide)acetyl)triacetic acid;
diethylenetriamine-N-(Gly_3(*p*-NCS-Phe-amide)acetyl)-N'-acetamide-N'',N''',N''''-triacetic acid;

1,4,7,10-tetraazacyclododecane-N-(Gly_2(*p*-NCS-Phe-amide)acetyl)-N',N'',N'''-triacetic acid;
1,4,7,10-tetraazacyclododecane-N-(Gly_4(*p*-NCS-Phe-amide)acetyl)-N',N'',N'''-triacetic acid;
1,4,7,10-tetraazacyclododecane-N-(Gly_5(*p*-NCS-Phe-amide)acetyl)-N',N'',N'''-triacetic acid;
1,4,7,10-tetraazacyclododecane-N-(Gly_6(*p*-NCS-Phe-amide)acetyl)-N',N'',N'''-triacetic acid;

1,4,7,10-tetraazacyclododecane-N,N'-di(Gly_3(*p*-NCS-Phe-amide)acetyl)-N'',N'''-diacetic acid;
1,4,7,10-tetraazacyclotridecane-N,N'-di(Gly_3(*p*-NCS-Phe-amide)acetyl)-N'',N'''-diacetic acid;
1,4,8,11-tetraazacyclotetradecane-N,N'-di(Gly_3(*p*-NCS-Phe-amide)acetyl)-N'',N'''-diacetic acid;
1,5,9,13-tetraazacyclohexadecane-N,N'-di(Gly_3(*p*-NCS-Phe-amide)acetyl)-N'',N'''-diacetic acid;
ethylenediamine-N,N'-di(Gly_3(*p*-NCS-Phe-amide)acetyl)-N'',N'''-diacetic acid;
diethylenetriamine-N,N'-di(Gly_3(*p*-NCS-Phe-amide)acetyl)-N''-acetamide-N''',N''''-diacetic acid;

1,4,7,10-tetraazacyclododecane-N-(Gly_3(*p*-NCS-Phe-amide)acetyl)-N'-acetamide-N'',N'''-diacetic acid;
1,4,7,10-tetraazacyclotridecane-N-(Gly_3(*p*-NCS-Phe-amide)acetyl)-N'-acetamide-,N'',N'''-diacetic acid;
1,4,8,11-tetraazacyclotetradecane-N-(Gly_3(*p*-NCS-Phe-amide)acetyl)-N'-acetamide-N'',N'''-diacetic acid;
1,5,9,13-tetraazacyclohexadecane-N-(Gly_3(*p*-NCS-Phe-amide)acetyl)-N'-acetamide-N'',N'''-diacetic acid;
ethylenediamine-N-(Gly_3(*p*-NCS-Phe-amide)acetyl)-N'-acetamide-N'',N'''-diacetic acid;
diethylenetriamine-N-(Gly_3(*p*-NCS-Phe-amide)acetyl)-N',N''-diacetamide-N''',N''''-diacetic acid;

1,4,7,10-tetraazacyclododecane-N-(Gly_3(*p*-SH-Phe-amide)acetyl)-N',N'',N'''-triacetic acid;
1,4,7,10-tetraazacyclododecane-N-(Gly_3(*p*-succinimidyl ester-Phe-amide)acetyl)-N',N'',N'''-triacetic acid;
1,4,7,10-tetraazacyclododecane-N-(Gly_3(*p*-chloroformyl-Phe-amide)acetyl)-N',N'',N'''-triacetic acid; and
1,4,7,10-tetraazacyclododecane-N-(Gly_3(*p*-chloroacetyl-Phe-amide)acetyl)-N',N'',N'''-triacetic acid.

Examples of radiolabeled chelates include:

$^{90}Y^{III}$-1,4,7,10-tetraazacyclododecane-N-(Gly_3(*p*-NCS-Phe-amide)acetyl)-N',N'',N'''-triacetate;
$^{90}Y^{III}$-1,4,7,10-tetraazacyclotridecane-N-(Gly_3(*p*-NCS-Phe-amide)acetyl)-N',N'',N'''-triacetate;
$^{90}Y^{III}$-1,4,8,11-tetraazacyclotetradecane-N-(Gly_3(*p*-NCS-Phe-amide)acetyl)-N',N'',N'''-triacetate;
$^{90}Y^{III}$-1,5,9,13-tetraazacyclohexadecane-N-(Gly_3(*p*-NCS-Phe-amide)acetyl)-N',N'',N'''-triacetate;
$^{90}Y^{III}$-ethylenediamine-N-(Gly_3(*p*-NCS-Phe-amide)acetyl) triacetate;
$^{90}Y^{III}$-diethylenetriamine-N-(Gly_3(*p*-NCS-Phe-amide)acetyl)-N'-acetamide-N'',N''',N''''-triacetate;

$^{111}In^{III}$-1,4,7,10-tetraazacyclododecane-N-(Gly$_3$(*p*-NCS-Phe-amide)acetyl)-N',N'',N'''-triacetate;
$^{111}In^{III}$-1,4,7,10-tetraazacyclotridecane-N-(Gly$_3$(*p*-NCS-Phe-amide)acetyl)-N',N'',N'''-triacetate;
$^{111}In^{III}$-1,4,8,11-tetraazacyclotetradecane-N-(Gly$_3$(*p*-NCS-Phe-amide)acetyl)-N',N'',N'''-triacetate;
$^{111}In^{III}$-1,5,9,13-tetraazacyclohexadecane-N-(Gly$_3$(*p*-NCS-Phe-amide)acetyl)-N',N'',N'''-triacetate;
$^{111}In^{III}$-ethylenediamine-N-(Gly$_3$(*p*-NCS-Phe-amide)acetyl) triacetate;
$^{111}In^{III}$-diethylenetriamine-N-(Gly$_3$(*p*-NCS-Phe-amide)acetyl)-N'-acetamide-N'',N''',N''''-triacetate;

$^{67}Cu^{II}$-1,4,7,10-tetraazacyclododecane-N,N'-di(Gly$_3$(*p*-NCS-Phe-amide)acetyl)-N'',N'''-diacetate;
$^{67}Cu^{II}$-1,4,7,10-tetraazacyclotridecane-N,N'-di(Gly$_3$(*p*-NCS-Phe-amide)acetyl)-N'',N'''-diacetate;
$^{67}Cu^{II}$-1,4,8,11-tetraazacyclotetradecane-N,N'-di(Gly$_3$(*p*-NCS-Phe-amide)acetyl)-N'',N'''-diacetate;
$^{67}Cu^{II}$-1,5,9,13-tetraazacyclohexadecane-N,N'-di(Gly$_3$(*p*-NCS-Phe-amide)acetyl)-N'',N'''-diacetate;
$^{67}Cu^{II}$-ethylenediamine-N,N'-di(Gly$_3$(*p*-NCS-Phe-amide)acetyl)-N'',N'''-diacetate;
$^{67}Cu^{II}$-diethylenetriamine-N,N'-di(Gly$_3$(*p*-NCS-Phe-amide)acetyl)-N''-acetamide-N''',N''''-diacetate;

$^{67}Cu^{II}$-1,4,7,10-tetraazacyclododecane-N-(Gly$_3$(*p*-NCS-Phe-amide)acetyl)-N'-acetamide-N'',N'''-diacetate;
$^{67}Cu^{II}$-1,4,7,10-tetraazacyclotridecane-N-(Gly$_3$(*p*-NCS-Phe-amide)acetyl)-N'-acetamide-,N'',N'''-diacetate;
$^{67}Cu^{II}$-1,4,8,11-tetraazacyclotetradecane-N-(Gly$_3$(*p*-NCS-Phe-amide)acetyl)-N'-acetamide-N'',N'''-diacetate;
$^{67}Cu^{II}$-1,5,9,13-tetraazacyclohexadecane-N-(Gly$_3$(*p*-NCS-Phe-amide)acetyl)-N'-acetamide-N'',N'''-diacetate;
$^{67}Cu^{II}$-ethylenediamine-N-(Gly$_3$(*p*-NCS-Phe-amide)acetyl)-N'-acetamide-N'',N'''-diacetate; and

$^{67}Cu^{II}$-diethylenetriamine-N-(Gly_3(*p*-NCS-Phe-amide)acetyl)-N',N''-diacetamide-N''',N''''-diacetate.

C. Purification of the radiolabeled chelate

The second step of prelabeling involves purification of the radiolabeled chelate. The aqueous chelating agent-radionuclide reaction mixture obtained in the first step of prelabeling may be purified by preparing an appropriate anion exchange medium, eluting the aqueous mixture through the medium, and collecting the eluent. Methods for preparing and using anion exchange media are well established and known to those skilled in the art. If necessary or desired, the eluent may be further concentrated by known methods.

The use of the well established technique of anion exchange chromatography to purify the radiolabeled chelate offers a number of practical advantages over other known methods of purification.

For example, the aqueous reaction mixture obtained in the first step of prelabeling may be purified simply and quickly by anion exchange chromatography to provide the purified compound in aqueous solution and in the absence of organic solvents. Consequently, there is no need to remove organic solvents from the purified material before proceeding with formation of the radiolabeled-chelating agent-ligand complex, as required by the existing prelabeling methods [see, for example, Linder *et al.*, 1991; Schlom *et al.*, 1991, *supra*]. The anion exchange chromatography purification step of the present invention saves time and thereby reduces the loss of radioactive potency and minimizes autoradiolysis.

Purification of the chelate by anion exchange chromatography can be significantly improved by selecting chelating agents and radionuclides with equal and opposite electrical charges. The resulting radiolabeled chelate is electrically neutral. The other important species in the chelation reaction mixture, such as excess

chelating agent, complexes containing differently charged metal ions, and quenching agent complexes, are negatively charged. Thus, the electrically neutral radiolabeled chelate of interest can be filtered quickly through an appropriately designed anion-exchange column in H_2O to separate them from anionic species. Purification can be further improved by selecting chelating agent/ radionuclide charges of -3/+3, so that chelates formed from adventitious cations, such as Ca^{+2} and Mg^{+2} are anionic.

Any appropriately prepared anion exchange material may be used to effect purification of the radiolabeled chelate. Commercially available (Sigma Chemical Company) anion exchange media include, for example, dextran-based anion exchange media such as DEAE Sephadex and QAE Sephadex, agarose-based anion exchange media such as DEAE Sepharose and Q Sepharose, cellulose-based anion exchange media, such as DEAE Sephacel, DEAE Cellulose, ECTEOLA Cellulose, PEI Cellulose, QAE Cellulose, and polystyrene-based anion exchange media, such as Amberlite and Dowex. Criteria for choosing and methods of preparation of anion exchange media are well established and known to those of skill in the art. At present, the preferred anion exchange medium is DEAE Cellulose prepared in acetate form.

D. Formation of the complex

The third step of prelabeling, conjugation, involves the formation of a radiolabeled-chelating agent-ligand complex (denoted herein variously as the chelate-ligand complex or the complex).

The term "ligand", as used herein, relates to compounds which may perform the role of targeting molecules, including, for example, targeting biomolecules such as antibodies, serum proteins, cell surface receptors, and tumor-specific targeting agents, such as bleomycin. The term "antibody" as used herein is generic

to polyclonal antibodies, monoclonal antibodies, chimeric antibodies, antibody fragments (particularly antibody binding fragments), recombinant single chain antibody fragments, and the like. Methods for the preparation of appropriate ligands, such as mAbs, are well established and known to those of skill in the art [See, for example, Fell *et al.*, 1992, *supra*].

Reaction conditions useful for the formation of the complex from the radiolabeled chelate and ligand, such as pH, temperature, salt concentration, and the like, will reflect the reactive pair of functional groups involved. In particular, reaction conditions useful for the formation of conjugates via functional groups present on proteins are well established and known to those of skill in the art. Typically, aqueous solutions or suspensions of the radiolabeled chelate and the ligand are mixed. The pH may be adjusted to optimize conditions for conjugation. For example, for reaction of the amino ($-NH_2$) group of a protein (for example, a mAb) with an isothiocyanato (-NCS) group, the pH may be adjusted to about 8 to 11, more preferably about 9.5, using, for example, a buffer such aqueous triethylamine. Furthermore, the temperature of the reaction mixture may be adjusted, for example, to 37°C for 1 hr, to accelerate conjugation. The reaction (incubation) time may be varied to optimize conjugation. Longer reaction times would lead to higher conjugation yields; however, for radioactivity levels appropriate for clinical use (≈100 millicuries, mCi), radiolysis will become important at longer times.

In the conjugation step, a high concentration of ligand (for example, mAb) is desired, so that each reactive functional group present on the radiolabeled-chelate (for example, isothiocyanate groups) will frequently encounter reactive functional groups on the ligand (for example, amino groups) with which to react. It may be desirable to concentrate the radiolabeled

chelate prior to the conjugation step to avoid dilution of the conjugation mixture, particularly when small amounts of radioactivity are used. Optimally, ligand concentrations as high as are practical are desired, for exmaple, about 10 to 500 mg/mL (the reaction mixture concentration of the chimeric mAb 16 ligand of Example 3 was about 20 mg/mL).

After conjugation, the complex may be separated from the reaction mixture and purified using known methods, for example, using a centrifuged gel-filtration column [Penefsky *et al.*, 1979, Methods Enzymol., 56, Part G:527-530; Meares *et al.*, 1984, Anal. Biochem., 142:68-78].

E. Use

One application of the radionuclide-labeled chelating agent-ligand complexes of the invention is for use in radioimaging. A variety of metal chelates, when conjugated with serum proteins, target-specific antibodies, or bleomycin can be localized in tumor or other target tissue to provide useful radioimaging images used in localizing tumors [Meares *et al.*, 1984; Goodwin *et al.*, 1979, Radiopharmaceuticals II, Proceedings of the Second International Symposium on Radiopharmaceuticals (V.J. Sodd, ed), New York, pp.275-284; De Reimer *et al.*, J. Lab. Comps. and Radiopharm., 18(10):1517; Meares *et al.*, 1976, Proc. Natl. Acad. Sci. U.S.A., 73(11):3803; Leung *et al.*, 1978, Int. J. Appl. Rad. Isot., 29:687; Goodwin *et al.*, 1981, J. Nucl. Med., 22(9):787]. For example, a composition comprising the radionuclide-labelled chelating agent-ligand complex and a pharmaceutically acceptable carrier is injected in a patient and allowed to localize, for example, in a tumor region(s). These regions are imaged using radioimaging equipment such as γ photon emission tomograph or a position emission computed tomograph. Various stratagems may be used to employed to enhance the image contrast which is achievable. For example, with a complex formed

with a serum protein, the image contrast can be improved by administering an anti-complex antibody following tumor uptake of the complex, to increase the rate of clearance of the complex from the bloodstream [Goodwin *et al.*, 1981, *supra*].

The radionuclide-labeled chelating agent-ligand complexes of the invention are also suitable for use as therapeutic agents based, for example, on the radiotherapeutic action of the radionuclide when localized in tumor tissue. Again, a composition comprising the radionuclide-labelled chelating agent-ligand complex and a pharmaceutically acceptable carrier is injected in a patient and allowed to localize, for example, in a tumor region(s).

F. Examples

The following examples further illustrate the invention. These examples are not intended to limit the invention in any manner.

Example 1

Preparation of the ^{90}Y-labeled chelating agent (**2**)

The prelabeling procedure is shown in Figure 1. (Bold letters in parentheses refer to the compounds shown in the figures). The bifunctional chelating agent (**1**) (denoted herein variously as DOTA-Gly_3-L-(*p*-isothiocyanato)-Phe-amide and 1,4,7,10-tetraazacyclododecane-N-(Gly_3(L-*p*-NCS-Phe-amide)acetyl)-N',N'',N'''-triacetic acid) was prepared by the method described by Li *et al.*, 1993, Bioconjugate Chem., 4:275-283. Carrier-free ^{90}Y (DuPont NEN) in 0.05 M HCl was dried in a heating block under N_2(g), and 100 μL of 20 mM (**1**) in 0.2 M $(CH_3)_4N^+$ acetate, pH 5.0, was added. The mixture was incubated at 37°C for 30 min, followed by the addition of 25 μL of 50 Mm DTPA in 0.1 M $(CH_3)_4N^+$ acetate, pH 6.0, for 15 min at room temperature (quenching; to complex any remaining free yttrium).

An anion-exchange column was prepared by filling a disposable 1 mL tuberculin syringe with 500 μL of DEAE (diethylaminoethyl) Cellulose anion-exchange resin (1 milliequivalent per dry gram; Sigma Chemical Company) and pre-spun for 3 min at ≈2000 g. The resin was converted to acetate form prior to use.

The solution was loaded onto the anion-exchange column, and the column was spun for 2 min at ≈2000 g. followed by elution with four 125 μL aliquots of H_2O by centrifugation at ≈2000 g for 2 min each. Most of the radioactive compound (**2**) (denoted herein variously as $^{90}Y^{III}$-DOTA-Gly_3-L-(*p*-isothiocyanato)-Phe-amide and $^{90}Y^{III}$-1,4,7,10-tetraazacyclododecane-N-(Gly_3(L-*p*-NCS-Phe-amide)acetyl)-N',N'',N'''-triacetate) was recovered in the first four fractions (see Table 1). One-step elution

with 0.5 mL of H_2O was performed for comparison, but it gave 18% lower recovery than stepwise elution with 0.5 mL of H_2O was performed for comparison, but it gave 18% lower recovery then stepwise elution.

All the eluted fractions were collected and concentrated to ≈15 μL with a speed-vac concentrator (Savant Instruments) without heating. It should be possible to avoid this step when larger amounts of radioactivity are used.

In the chelation step, the yield after anion-exchange was typically >70% of the starting radioactivity. Particularly for ^{90}Y solutions, the levels of metal impurities appear to vary with each batch of carrier-free radiometal. The identity of these impurities is difficult to determine, but most common metal contaminants are divalent ions. Pre-labeling deals with the impurity problem by using a large excess of chelating agent, and then removing the excess. This is preferable to using a large excess of chelate-tagged mAb conjugate, which cannot be fractionated later to remove unwanted contaminants. Obviously, pre-labeling does not eliminate trivalent metal complexes from the product.

Example 2

Preparation of the ^{111}In-labeled chelating agent (**2**)

^{111}In-labeled chelate (**2**) was prepared by the general procedure of Example 1.

Example 3

Preparation of the radionuclide-labeled chelating agent-targeting molecule complex (3)

Concentrated solutions of the labeled chelating agent of Example 1 (and alternatively of Example 2) were mixed with 1 mg of chimeric mAb 16 (18 μL, 56 mg/mL; Oncogen/Bristol-Myers) [Fell *et al.*, 1992, J. Biol. Chem., 267:15552-15558] in 0.1 M $(CH_3)_4N^+$ phosphate, pH 9.0. The pH was adjusted to 9.5 using aqueous 2.0 M triethylamine. The mixture was incubated at 37°C for 1 hr and compound (3) was isolated using a centrifuged gel-filtration column [Penefsky *et al.*, 1979; Meares *et al.* 1984, *supra*]. Yields are listed in Table 1.

Table 1

Results for DOTA-Peptide Radiolabeling and Conjugation

Radio-nuclide	Starting Radioactivity (volume)	Recovery for Step 2† (radio-labeling)	Recovery for Step 3† (conju-gation)	Overall Recovery †
^{90}Y	2.1-3.9 mCi (2-5μL)	80%±5%	40%±2%	30%±4%
^{111}In	4.9-6.2 mCi (12-30μL)	70%±9%	73±3%	42%±4%

† Average recovered radioactivity ± standard deviation, for ≥3 runs.

The radiochemical purity of both ^{90}Y-and ^{111}In-labeled immunoconjugates (3) was determined to be >95% by gel filtration HPLC cellulose acetate electrophoresis, and silica gel TLC [Meares *et al.*, 1984, *supra*]. A solid-phase radioimmunoassay [DeNardo *et al.*, 1986, Nucl. Med. Biol., 13:303-310] was performed using ^{125}I-labeled chimeric L6 as a standard. The immunoreactivity of ^{90}Y-DOTA-Gly_3-L-Phe-amide-thiourea-chimeric L6 was 107 ± 5% relative to ^{125}I-labeled antibody.

Example 4

Biodistribution of the radionuclide-labeled chelating agent-targeting molecule complex

To examine the properties of the conjugate in vivo, ^{90}Y-labeled compound (3) was injected into HBT tumor-bearing nude mice [Hellström *et al.*, 1986, Cancer Res., 46:3917-3923] for organ distribution and tumor uptake studies. The results of these animal studies (summarized in Figure 2) showed that the radioactivity level in the liver varied from 6.4±1.5% I.D./g on the first day to 5.4±1.5% I.D./g on the third day to 4.6±1.9% I.D./g on the fifth day. The units I.D./g indicate percent of injected dose per gram of tissue. The tumor uptake was 17.5±8.0% I.D./g on day 1, 18.0± 8.0%I.D./g on day 3 and 13.8%±5.2%I.D./g on day 5. The bone uptake was 2.1±0.3%I.D./g, 2.0±0.5±%I.D./g, and 1.8±0.2%I.D./g on days 1, 3, and 5. The levels of radioactivity in liver and bone are satisfactorily low [Deshpande *et al.*, 1990, *supra*], and the tumor uptake is good.

All publications, patents, and patent applications cited in this specification are herein incorporated by reference as if each individual publication, patent, or patent application were specifically and individually indicated to be incorporated by reference.

Claims

1. A method for preparing a radionuclide-labeled chelating agent-ligand complex comprising:

(a) reacting a chelating agent that has a chelating group and at least one pendant linker group that is capable of covalently binding a ligand with a radionuclide to form a radionuclide chelate;

(b) purifying the radionuclide chelate from the reaction mixture of step (a) by anion exchange chromatography; and

(c) reacting the purified radionuclide chelate of step (b) with the ligand to form said complex.

2. The method of claim 1 wherein the electrical charges of the chelating agent and the radionuclide are equal and opposite.

3. The method of claim 2 wherein the electrical charge of the radionuclide is +3 and the electrical charge of the chelating agent is -3.

4. The method of claim 3 wherein the radionuclide is ^{90}Y or ^{111}In.

5. The method of claim 1 wherein the chelating group is a macrocyclic group.

6. The method of claim 1 wherein in the chelating group is a polyazamacrocyclic group or a polyoxamacrocyclic group.

7. The method of claim 1 wherein the chelating group is derived from 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid; 1,4,7,10-tetraazacyclotridecane-N,N',N'',N'''-tetraacetic acid; 1,4,8,11-tetraazacyclotetradecane-N,N',N'',N'''-tetraacetic acid; or 1,5,9,13-tetraazacyclohexadecane-N,N',N'',N'''-tetraacetic acid.

8. The method of claim 1 wherein the chelating agent is N-substituted 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid wherein the substituent is $(Gly)_3$-L-(*p*-isothiocyanato)-Phe-amide and the radionuclide is ^{90}Y or ^{111}In.

9. The method of claim 1 wherein the ligand is an antibody.

10. The method of claim 1 wherein the ligand is a monoclonal antibody.

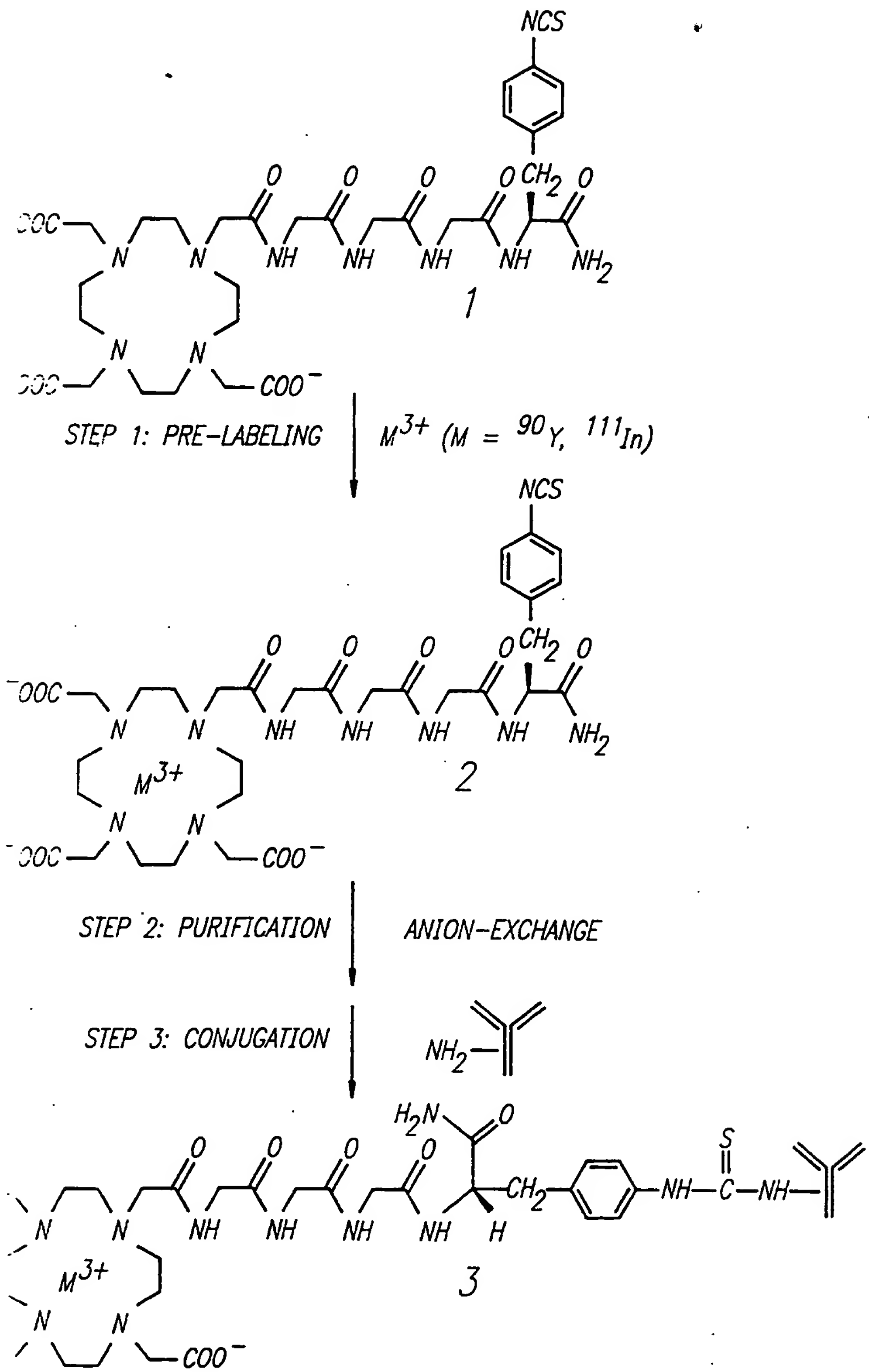
11. The method of claim 4 wherein the ligand is an antibody.

12. The method of claim 4 wherein the ligand is a monoclonal antibody.

13. The method of claim 8 wherein the ligand is an antibody.

14. The method of claim 8 wherein the ligand is a monoclonal antibody.

FIG. 1


NCS
O
O
O
O
CH_2
O
^{-}OOC
N
N
NH
NH
NH
NH
NH_2
1
N
N
^{-}OOC
COO^-
STEP 1: PRE-LABELING
M^{3+} (M = ^{90}Y, ^{111}In)
NCS
CH_2
^{-}OOC
M^{3+}
NH_2
2
COO^-
STEP 2: PURIFICATION
ANION-EXCHANGE
STEP 3: CONJUGATION
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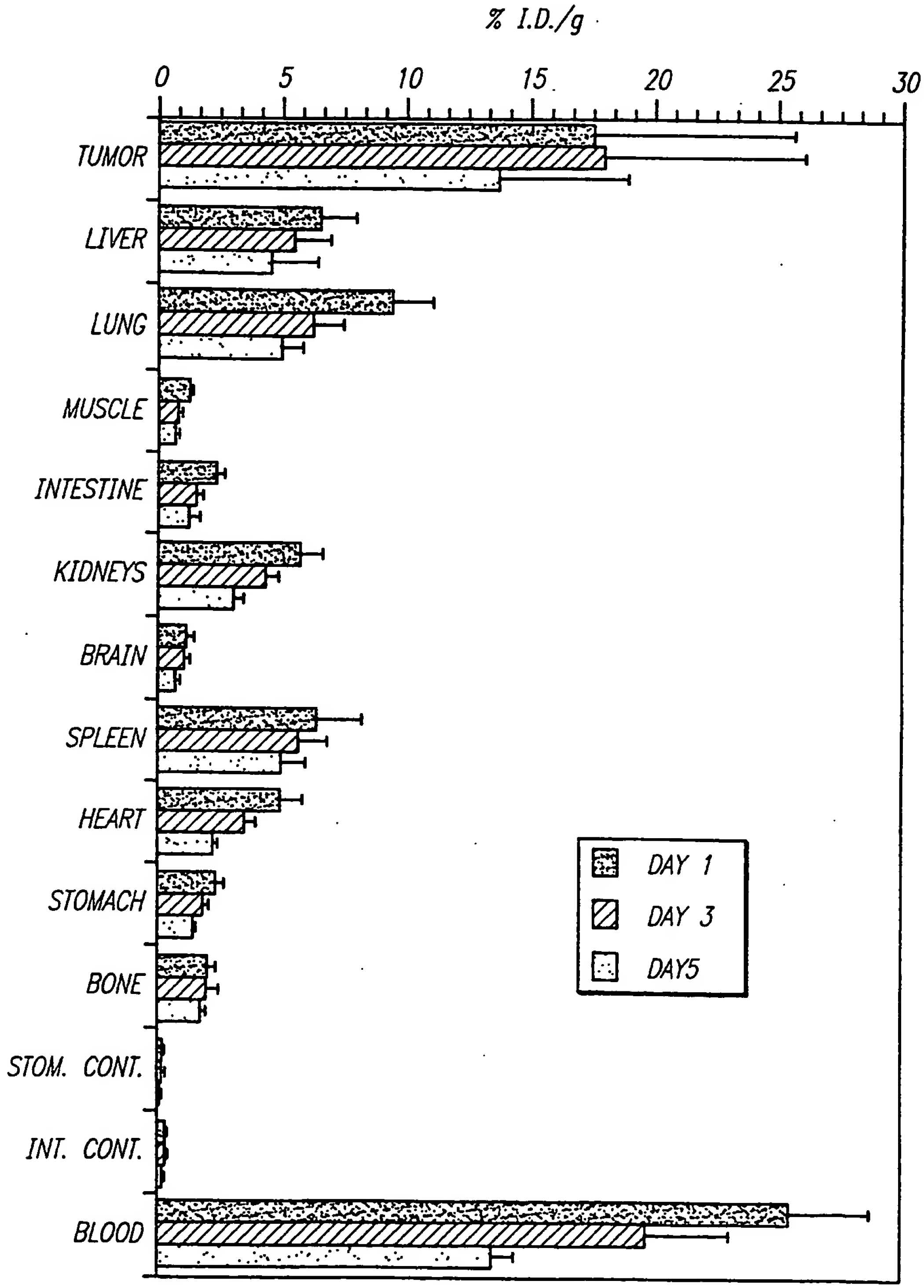
S
CH_2
NH—C—NH
H
3
M^{3+}
COO^-

FIG. 2


% I.D./g
0
5
10
15
20
25
30
TUMOR
LIVER
LUNG
MUSCLE
INTESTINE
KIDNEYS
BRAIN
SPLEEN
HEART
STOMACH
BONE
STOM. CONT.
INT. CONT.
BLOOD
DAY 1
DAY 3
DAY5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/03722

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 51/08, 51/10
US CL :424/1.53
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/1.53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

none

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A, 89/12631 (Cheng et al.) 28 December 1989, see page 7, lines 5-24, page 39, lines 25-29 and pages 42-43 as well as page 45, lines 1-10; see also Examples IX-XII on pages 128-131.	1-14
X --- Y	US, A, 5,284,644 (Kruper, Jr., et al.) 08 February 1994, see Examples 20-21 in columns 21-22.	1-3, 5-7, 9-10 --- 4, 8, 11-14
Y	US, A, 5,217,704 (Johnson et al.) 08 June 1993, see background in columns 1-8.	1-14
Y	US, A, 5,006,643 (Fazio et al.) 09 April 1991, see columns 1-2.	1-14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 JUNE 1995	12 JUL 1995

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cancer Research, Volume 51, issued 01 June 1991, Schlom et al., "Monoclonal Antibody-based Therapy of a Human Tumor Xenograft with a 177 Lutetium-labeled Immunoconjugate," pages 2889-2896, especially page 2890.	1-14
X --- Y	Analytical Biochemistry, Volume 148, issued 1985, Moi et al., "Copper Chelates as Probes of Biological Systems: Stable Copper Complexes with a Macrocyclic Bifunctional Chelating Agent," pages 249-253, especially page 251.	1, 5-7, 9-10 --- 2-4, 8, 11-14
Y	Chemical Society Reviews, Volume 19, issued 1990, Parker, "Tumour Targeting with Radiolabelled Macrocycle-Antibody Conjugates," pages 271-291, especially pages 271-273.	1-14